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To better understand the role of DNA replication in breast cancer, it is essential to examine the machinery that carries out the DNA synthetic process. Our laboratory has successfully purified a complex of proteins from breast cells that is fully competent to carry out T-antigen dependent, SV40 origin specific DNA replication *in vitro*, which we have termed the DNA synthesome. Analysis of the constituent proteins of the DNA synthesome of malignant and non-malignant breast cells by two-dimensional polyacrylamide gel electrophoresis (2D PAGE) has uncovered a modification in an essential DNA replication protein, proliferating cell nuclear antigen (PCNA). 2D PAGE analysis revealed that PCNA of malignant breast cells resolves as two distinct species, an unmodified form and a modified form, while PCNA present in non-malignant cell resolves exclusively as one form, the modified form. PCNA functions by forming a trimeric "sliding clamp" that encircles the DNA and interacts with the DNA polymerases δ and ε. Polymerase δ carries out leading strand DNA synthesis, and although a role for polymerase ε has not yet been ascribed, it has been hypothesized to function in DNA repair. Another protein that interacts with PCNA is $p21^{WAF1/CIP1/SDI1}$. P21 is a CDK inhibitor that, when induced by p53 in response to DNA damage, binds PCNA and effectively competes away polymerase δ leading to the efficient inhibition of DNA replication. This inhibition impedes the replication of damaged DNA and theoretically allots time for the cell to repair its damaged DNA. Therefore, any alterations of the PCNA molecule could potentially abrogate p21 binding leading to replication of damaged DNA and/or insufficient time for DNA repair. It is our goal to study the interaction of p21 with the modified and non-modified forms of PCNA and to investigate any functional consequences alterations in PCNA/p21 binding may have on DNA replication, DNA repair, and DNA replication fidelity.

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INTRODUCTION

To better understand the role of DNA replication in breast cancer, it is essential to examine the machinery that carries out the DNA synthetic process. Our laboratory has successfully purified a complex of proteins from breast cells that is fully competent to carry out T-antigen dependent, SV40 origin specific DNA replication *in vitro* (Li *et al.*, 1984) which we have termed the DNA synthesome (Malkas *et al.*, 1990; Coll *et al.*, 1997). Analysis of the constituent proteins of the DNA synthesome of malignant and non-malignant breast cells by two-dimensional polyacrylamide gel electrophoresis (2D PAGE) has uncovered a modification in an essential DNA replication protein, proliferating cell nuclear antigen (PCNA) (Bechtel *et al.*, 1998). 2D PAGE analysis revealed that PCNA of malignant breast cells resolves as two distinct species, a unmodified form and a modified form, while PCNA present in non-malignant cells resolves exclusively as one form, the modified form. PCNA functions by forming a trimeric "sliding clamp" that encircles the DNA and interacts with the DNA polymerases δ and ε (Krishna *et al.*, 1994; Zhang *et al.*, 1998;). Polymerase δ carries out leading strand DNA synthesis, and although a role for polymerase ε has not yet been ascribed, it has been hypothesized to function in DNA repair (Hindges *et al.*, 1997). Another protein that interacts with PCNA is $p21^{WAF1/CIP1/SDI1}$ (Li *et al.*, 1994; Luo *et al.*, 1995). P21 is a CDK inhibitor that, when induced by p53 in response to DNA damage, binds PCNA and effectively competes away polymerase δ leading to the efficient inhibition of DNA replication (Waga *et al.*, 1994; Podust *et al.*, 1995Warbrick *et al.*, 1997). This inhibition impedes the replication of damaged DNA and theoretically allots time for the cell to repair its damaged DNA. Therefore, any alterations of the PCNA molecule could potentially abrogate p21 binding leading to replication of damaged DNA and/or insufficient time for DNA repair. It is our goal to study the interaction of p21 with the unmodified and modified forms of PCNA and to investigate any functional consequences alterations in PCNA/p21 binding may have on DNA replication, DNA repair, and DNA replication fidelity.

Progress Report

Purification and separation of the two forms of PCNA

Two dimensional polyacrylamide gel electrophoresis (2D PAGE) and electroelution of the different forms of PCNA in MCF7 cells was performed in an attempt to purify the two forms of PCNA. Initially, MCF7 cells were fractionated to a clarified nuclear extract and post-microsomal (NE/S3) fraction and placed onto a Phosphocellulose column. Phosphocellulose dissociated the synthesome multiprotein complex and resolves PCNA from the DNA polymerases and other synthesome associated DNA replication proteins. The column eluate fraction containing PCNA was then passed over a hydrophobic Phenyl Sepharose column followed by anion exchange chromatography (Q Sepharose). Purified PCNA was then loaded onto 2D PAGE and resolved. One of the gels was silver stained and analyzed using the Melanie II software. Analysis predicted the general location of the PCNA species and empirically predicted the isoelectric points (pIs) of the two species. The pIs for PCNA were 4.74 for the unmodified (cancer specific) form, which is close to the theoretical pI for PCNA (4.56), and 6.96 for the modified (non-malignant) form. The unmodified form and modified form of PCNA were then excised from four parallel 2D PAGE gels and electroeluted into 25mM HEPES buffer pH 7.2 using a Mini Whole-Gel Eluter (Bio-Rad). The presence of PCNA in the electroeluted fractions was determined by slot blotting (Life Technologies, Inc.), and the fractions containing PCNA were subsequently tested by 2D PAGE. The 2D gels confirmed the presence of the unmodified form in the electroelute; however, 2D analysis of the modified form of PCNA in the electroelute demonstrated the exclusive presence of the cancer-specific form. It was concluded from these data that the modification of PCNA was lost in either the electroelution or, more probably, the second dimension SDS PAGE step. Because of the apparent loss of modification, new approaches to separating the two forms of PCNA have begun to be developed.

In order to separate the two forms of PCNA, an alternative chromatographic protocol will be employed. The Q-Sepharose chromatography has been replaced with cation exchange chromatography (SP Sepharose). PCNA eluted off the Phenyl Sepharose column will be dialyzed into 50mM potassium phosphate buffer with a pH of 3.0. Because the pH of the buffer is below the pIs for the two forms of PCNA, the molecules will have a positive character and bind to the SP Sepharose. The unmodified form can now be eluted from the column in 50mM potassium phosphate pH 5.75, and because the pH of this buffer is still below the pI of the modified form, it will still bind the column and not be eluted. The modified form can then be eluted into 50mM potassium phosphate pH 8.0. This will enable us to better study the unmodified and modified forms of PCNA and their interactions with p21.

Cloning PCNA into a protein expression vector

In order to study PCNA and its modification(s) we are going to express and modify the protein *in vitro*. To do this we cloned the PCNA from MCF7 cells. The cDNA sequence encoding the entire PCNA translation unit was prepared using Superscript Preamplification System First Strand DNA Synthesis (Life Technologies, Inc.) followed by cloning of the amplified cDNA into the vector pCR2.1 (InVitrogen) according to the manufacturers instructions. Total RNA was isolated using Trizol reagent (Life Technologies, Inc.). Second strand DNA synthesis was carried

out by priming the first strand cDNA with oligonucleotide 5'-GCGTTGTTGCCACTCCGC-3' on the 5' end of the cDNA and 5'-GCAGTTCTCAAAGAGCTTAG-3' on the 3' end of the cDNA and amplifying the primed first strand using reverse transcriptase PCR.

Subcloning of the cDNA was done using the pDUAL expression and cloning kit according to the manufacturer's protocol (Stratagene). Briefly, Eam1104I restriction sites were engineered onto the 5' ends of the PCR primers 5'-ATGTTCGAGGCGCGCCTGGTCCAG-3' and 5'-AGATCCTTCATCCTCGATCTTGGGAGC-3' and the amplified PCNA cDNA was inserted into the pDUAL expression vector. Purification of PCNA is accomplished using Calmodulin Affinity Resin (Stratagene) which specifically binds a Calmodulin Binding Protein (CBP) tag fused to the C-terminus of the protein.

2D PAGE, tryptic digestion of PCNA, and MALDI-TOF mass spectrometry

A new approach has been employed to identify the amino acid sequence harboring the PCNA modification. MCF7 cells were fractionated and PCNA was purified through a Q Sepharose chromatography as outlined in above. The two forms of PCNA were then resolved on 2D PAGE and visualized by silver staining using Silver Stain Plus (Bio-Rad). The spots corresponding to PCNA are then excised from the gel and digested with trypsin. The resultant peptide fragments are then resolved using an MALDI-TOF mass spectrometer in the negative ion, reflectron mode. The molecular masses of the peptide fragments obtained by MALDI-TOF mass spectrometry are then used to search protein sequence databases available on the inter-net. Sites of post-translational modification can then be determined by increases in the apparent molecular masses of the peptide fragment(s). The mass difference of these shifted peptide fragments will give insight into the identity and location of the modification(s) on PCNA.

Using a Voyager DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, Inc.) we were able to resolve peptide fragments derived form PCNA excised from 2D PAGE, and by searching the proteomics database Protein Prospector (UCSF), we were able to identify the protein as PCNA by its tryptic digest pattern. Further work will enable us to resolve the modified fragment(s) identifying the location and nature of the modification(s).

***In vitro* transcription and translation of GST-p21**

To study the interactions of p21 with the two forms of PCNA, GST- p21 protein was produced *in vitro* using *E. coli* T7 S30 Extract System for Circular DNA (Promega) for use in Far Western blotting experiments (see below). GST-p21 was labeled by addition of ^{3}H-leucine to the *in vitro* transcription and translation reaction and purified on a Glutathione Sepharose column (Amersham Pharmacia). Presence of *in vitro* protein product was confirmed by SDS-PAGE and autoradiography.

Co-immune precipitation, GST pull-down assays, and Far Western blotting

To demonstrate that the modification(s) on PCNA effects its ability to interact with p21, three different experimental approaches were taken. First, the interaction of p21 with the two forms of PCNA was examined by co-immune precipitation. MCF7 cells were fractionated to a NE/S3 and

incubated with monoclonal anti-p21 antibody (DF10, Oncogene Research) at 4°C for 2 hours. The antibodies were then bound to Protein-A-Agarose (Oncogene Research) and washed. The precipitate was then resolved by 2D PAGE, transferred to nitrocellulose, and Western blotted using anti-PCNA antibody (PC10, Oncogene Research) (Figure 4). Secondly, GST pull-down experiments were performed. GST-p21 was purified from inclusion bodies *E. coli* BL21 (DE-3) cells (Stratagene) and purified using Glutathione Sepharose. MCF7 NE/S3 was then added to the GST-p21 conjugated Glutathione Sepharose beads and incubated at 4°C for 2h. The beads were washed and loaded onto 2D PAGE. The 2D gels were transferred to nitrocellulose and Western blotted with PCNA antibody. The co-immune precipitation and GST pull-down experiments showed similar results. They both demonstrated the presence of PCNA; however, the precipitated PCNA has a pI in-between that of the two forms of PCNA found in the MCF7 NE/S3 control. Another assay used to elucidate binding of p21 to PCNA was Far Western blotting. Far Western blotting utilizes a Western blotting approach that uses a labeled p21 instead of PCNA antibody to detect PCNA. MCF7 NE/S3 was first resolved on 2D PAGE and transferred to PVDF membrane. The proteins immobilized on the membrane were then denatured in buffer containing 6M guanidine HCl for 1h, slowly refolded in 3M guanidine HCl and step-wise down to 0.187 M and finally into buffer. The re-natured membranes were incubated with labeled GST –p21 (see above) over night at 4°C. The membranes were washed three times with buffer, dried, sprayed with En^{3}Hance (New England Nuclear) and exposed to autoradiography at –80°C. Despite numerous attempts, we were unable to detect p21 binding to PCNA on 2D gels by Far Western blotting. Although spots are visible on the autoradiographs, indicating p21 binding, none of the spots overlap PCNA visualized by Western blotting of 2D gels run in parallel. Due to the inability of these experiments to demonstrate that the modification(s) on PCNA effects p21 binding, we have begun to take a new experimental direction.

New directions for analysis of the interaction between p21 and the two forms of PCNA

To definitively show that the modification(s) on PCNA effects its ability to interact with p21, we have now begun to use BIAcore 2000 surface plasmon resonance (SPR) mass spectrometer (BIAcore, Inc). Briefly, the unmodified form and modified form of PCNA will be bound to BIAcore CM-5 chips by carbidamide linkage. Next, varying concentrations of p21 will then be passed over the chip surface and binding events will be detected by changes in molecular mass. The data collected for the binding of p21 to the unmodified and modified forms of PCNA will then be converted into dissociation constants (K_d) using BIAevaluation software version 2.1 (BIAcore, Inc.) and Scatchard analysis. In addition, it has also come to our attention that the bacterially produced p21, which has been utilized in previously published BIAcore experiments, would not be optimal for K_d determination due the inevitable mis-folding of a percentage of p21 during the inclusion body purification. To address this issue we will use insect *sf9* cells to produce our recombinant p21. The p21 cDNA has been amplified using the PCR primers 5'-ATGTCAGAACCGGCTGG-3' with a 5' BamHI site and 5'-GGGCTTCCTCTTGGAGA-3' with a 5' EcoRI site and a 6X HIS tag. The amplified fragment was then sub-cloned into pFastBac1 expression vector using the Bac-TO-Bac Baculovirus Expression System (Life Technologies, Inc.), p21will be stably transfected into the *sf9* cells, which will allow us to generate a purified, properly folded recombinant protein leading to a more reliable K_d value.

Key Research Accomplishments

- **Performed 2D PAGE and electroeluted PCNA**
- **Tryptic digested PCNA excised from 2D PAGE and analyzed peptide fragments by MALDI-TOF mass spectrometry**
- **Cloned PCNA into pDUAL plasmid expression vector**
- **Co-immune experiments using anti-p21 antibody and analysis of precipitated PCNA by 2D PAGE**
- **Precipitation of PCNA by GST-p21 and analysis by 2D PAGE**
- ***In vitro* transcription, translation and purification of GST-p21**
- **Far Western blotting of 2D PAGE with ^{3}H labeled GST-p21**

Reportable Outcomes

Abstracts

Hoelz, D.J., Park, M., Dogruel, D., Bechtel, P., Sekowski, J., Xiang, H.Y., Hickey, R.J., Malkas, L.H. (2000): Analysis of a Malignant Cell's DNA Replication Apparatus by Mass Spectrometry. Scientific Proceeding of the 91st Annual Meeting of the American Association for Cancer Research. 41: 847.

Papers

Hoelz, D.J., Bechtel, Hickey, R.J., Malkas, L.H. (2000): Purification of the Malignant Form of Proliferating Cell Nuclear Antigen from Breast Cancer cells. Manuscript in preparation.

Conclusions

- **The unmodified form of PCNA has a pI of 6.96**
- **The modified form of PCNA has a pI of 4.76**
- **The modification to PCNA is apparently lost upon 2D PAGE and its susequent electroelution from the gel**
- **The two forms of PCNA can be purified by ion-exchange chromatography taking into account their pIs**
- **Co-immune precipitation of PCNA with anti-p21 antibody produces a species of PCNA with an apparent pI different than that of the unmodified and modified forms**
- **GST-p21 pull down assays also produce a species of PCNA with a different pI identical to that of the co-immune precipitation**
- **Labeled recombinant GST-p21 is unable to bind PCNA in the Far Western experiments, which could be a function of the loss of modification mentioned above**

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